Identification of a novel human homolog of the *Drosophila* dlg, P-dlg, specifically expressed in the gland tissues and interacting with p55

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Abstract We have identified a novel human homolog of the *Drosophila dlg* tumor suppressor gene, termed *P-dlg*, which has been mapped at chromosome 10q23. Unlike other human *dlg* homologs, *P-dlg* is expressed in placenta and various gland tissues but not in brain. The P-dlg protein is localized at the plasma membrane and cytoplasm, and it is expressed in the gland epithelial cells in normal prostate tissue but not in prostate cancer cell lines. Furthermore, we identified interaction between P-dlg and p55 palmitoylated membrane protein by yeast two-hybrid screening. These findings suggest that P-dlg forms a complex with p55 at the plasma membrane and plays roles in maintaining the structure of epithelial cells and transmitting extracellular signals to the membrane and cytoskeleton, which may negatively regulate cell proliferation.

Key words: P-dlg; MAGUK family protein; p55; Chromosome 10q23

1. Introduction

The lethal(1)-discs large (*dlg*) gene was originally identified based on its ability to act as a tumor suppressor in *Drosophila* [1]. The recessive mutant of this gene leads to disruption of normal cell-cell adhesion and neoplastic overgrowth of the imaginal disc epithelium. The protein encoded by the *dlg* gene was found to be localized in the apical-lateral membrane of epithelium cells at the tight junction. This evidence suggests that the wild-type dlg protein plays a role in composing and maintaining normal epithelium structure [2].

Several vertebrate homologs of the *dlg* gene have been identified and characterized in recent years, and they have been categorized into a new protein family termed MAGUK (membrane-associated guanylate kinase homolog) [3]. The PSD95, which is also known as SAP90 [4,5], is predominantly expressed in the brain, where it localizes in the postsynaptic membrane and presynaptic axon terminals of inhibitory neurons. PSD95/SAP90 binds directly to the cytoplasmic tail of both Shaker-type voltage-gated K^+ channels and the NR2 modulatory subunit of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors [6,7]. Two other dlg-related MAGUK proteins, chapsyn 110 [8] and NE-dlg/SAP-102 [9–11], have also been shown to interact with NMDA receptors. These proteins contain three distinct domains: an N-terminal segment comprising one or three copies of a 80–90 amino acid motif called PDZ (PSD-95, Dlg, ZO-1) domain, a *src* homology 3 (SH3) domain, and a region with high similarity to guanylate kinases (GUK) [12]. The PDZ domain has been shown to be a module for interacting with various cellular proteins. SAP90/PSD-95, hdlg-1/SAP97 [13,14] and NE-dlg/SAP102 were found to bind to the carboxy-terminal tail of the NMDA receptor through their PDZ domain. Furthermore, PDZ domains of NE-dlg/SAP102 and hdlg-1/SAP97 were shown to interact with APC tumor suppressor protein [9,15]. Both the NMDA receptor and APC protein have a threonine/serine-X-valine (T/S-X-V) motif at their carboxy-terminal end, and this motif has been proved to specifically interact with PDZ domains. Analysis of the crystal structure for the PDZ domain has revealed that it forms a carboxylate binding loop utilizing its β-sheets containing the sequence Gly-Leu-Gly-Phe (GLGF) [16] which is highly conserved in most of the PDZ domains.

The GUK domain is homologous to the domain of the yeast guanylate kinase which is an enzyme that catalyzes the transfer of phosphate from ATP to GMP, forming GDP. However, the dlg-related GUK has been found to have no guanylate kinase activity. Recently, DAP-1, a novel protein which interacts with GUK domains of hdlg-1 and PSD95, was identified [17]. Thus, the GUK domain is also considered to contribute to protein-protein interaction.

All *Drosophila* dlg homologs identified to date (SAP90/PSD95, hdlg1/SAP97, and NE-dlg/SAP102) were found to be highly expressed in neuronal cells and to be important components for forming synaptic structure. In this study, we have identified and characterized a novel human homolog of *Drosophila* dlg, termed P-dlg, which is highly expressed in placenta and various gland tissues, but not in brain. P-dlg has three PDZ domains, which do not have the conserved GLGF motif. These lines of evidence led us to hypothesize that P-dlg has a unique function distinct from other dlg homologs. Furthermore, we have identified interaction between P-dlg and p55 [18–21], which is a member of the MAGUK family, using the yeast two-hybrid screening system. The biological significance of this novel dlg homolog will be discussed.

2. Materials and methods

2.1. EST database screening and PCR-based full length cDNA cloning

We searched for human homologs of the *dlg* gene by screening the database of the expressed sequence tag (dbEST) at the National Center for Biotechnology Information (NCBI). The method for PCR-based full length cDNA cloning from EST clones was reported previously [22]. For amplification of the 5′ region, the first PCR was

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performed using only primer P1 (5′-GCATCCAAGGCGAGCAG-GTCTTT-3′) to amplify single-strand cDNA from a human fetal brain cDNA library (Clontech, Palo Alto, CA) in a 25 μl reaction volume for 50 cycles. The first PCR product was used as a template in the second run, where P2 (5′-TGCAGCCGCTGACAGCGTCT-TTGT-3′) and T3 (5′-ATTAACCCTCACTAAAG-3′) were utilized as primers to amplify the 5′ region cDNA. For amplification of the remaining 5′ region, P3 (5′-CTTCGTGAACTCCTCAGGGCGGTA-3′) and P5 (5′-ACTCGAAGAACCCAGGCGGGCGCT-3′) were used for the first PCR, and P4 (5′-CACCTTCAGGCGGACGC-CATCCCT-3′), P6 (5′-CAGTGAGTTGGACCCATGGGTGGCT-CT-3′) and T7 (5′-AATACGACTCACTATAG-3′) were used for the second PCR. The PCR fragments were direct-sequenced using the same primers.

2.2. Northern blot analysis

Northern blots derived from multiple human tissues and cancer cell lines containing 2 μg of poly(A)$^+$ RNA per lane were obtained from Clontech. The membrane was probed with a 1.1 kbp cDNA fragment of *P-dlg* that had been labeled with [α-^{32}P]dCTP as previously described [9].

2.3. Radiation hybrid (RH) mapping of P-dlg gene

PCR was performed to detect the *P-dlg* gene in the Genebridge 4 RH Screening Panel using a set of primers (S int-1: 5′-GTACA-GAAAATCCCAGTC-3′ and AS int-2: 5′-TCAAGGTATTAACT-CGCAG-3′) which were designed based on a partial genomic sequence of the *P-dlg* gene. The primers were expected to amplify a 125 bp PCR product from human genomic DNA as a template. PCR was carried out as previously described [9]. The PCR results of the RH Panel were sent to the Whitehead Institute/MIT Center for Genome Research via the internet for mapping of the genes relative to the radiation hybrid map of the human genome [23].

2.4. Production of anti-P-dlg polyclonal antibody

An antibody against a unique protein sequence in P-dlg was raised by subcutaneous immunization of rabbits with a synthetic peptide (KSAKHIKEKEQRDPIYLRDKVTQRH) which was coupled to keyhole limpet hemocyanin. The antibody was affinity-purified on a column containing the peptide cross-linked to Sepharose.

2.5. Construction of the P-dlg expression plasmid

The full length ORF of the *P-dlg* cDNA (2577 bp) was amplified by PCR from the λZAP human fetal brain cDNA library using *rTth* DNA polymerase and a set of primers: P-top (5′-TGGACGGATC-CATGCACGCATCACCCCCTCGCAAGG-3′) containing a *Bam*HI site (underlined) and the primer P-stop (5′-AGCTCTAGACTA-GAGCGGGCAGGCTGGAAT-3′) containing a *Xba*I site (underlined). The PCR fragment was digested with *Bam*HI and *Xba*I, and ligated into a pCGN expression vector. To express HA epitope-tagged P-dlg, the pCGN-P-dlg was transfected into COS-7 cells by the liposome-mediated gene transfer method.

2.6. Western blotting

Lysates of cultured cells (HeLa S3 cell; human cervix adenocarcinoma cell lines, PC-3 and Du-145 cell; human prostate tumor cell lines) and frozen tissues were separated on an 8% polyacrylamide gel and transferred to a nitrocellulose filter with a constant current of 140 mA for 2 h. The filters were probed with 1:1000 diluted anti-HA antibody (12CA5) and 1:500 diluted anti-P-dlg antibody using the method previously described [9].

2.7. Immunohistochemical analysis

Frozen tissues were cut into sections 6 μm thick and fixed in 10% buffered formalin for 15 min. After fixation, immunoperoxidase staining of the tissue sections was performed as previously described [9].

2.8. Immunofluorescence staining and confocal laser scanning microscopic (CLSM) analysis

HeLa S3 cells were seeded on glass-bottom culture dishes (MatTek Corp.) and incubated for 24 h at 37°C, then washed twice with PBS and fixed in 4% PFA for 10 min followed by 0.2% Triton X-100 for 5 min. The cells were incubated with anti-P-dlg polyclonal clonal antibody followed by treatment with FITC-conjugated secondary antibody. After being washed with PBS, they were mounted in 50% glycerol, and visualized with a confocal microscope (TCS 4D, Leica, Nussloch, Germany) equipped with an argon gas laser and appropriate filter sets to allow the simultaneous recording of fluorescein.

2.9. Yeast two-hybrid screening

The partial P-dlg cDNA, which included three PDZ repeats and the SH3 region, was obtained by PCR using a set of primers (5′-TGGACGCTAGCATGCACGCATCACCCCCTCGCAAGG-3′ and 5′-GACCTAGGATCCAAGGCGAGCAGGTCTTTCCCGT-3′) containing *Nhe*I and *Bam*HI cloning sites (underlined), respectively. The PCR product was double digested with *Nhe*I and *Bam*HI and subcloned into the pBTM116HA vector as a fusion to the LexA DNA-binding domain. The resultant plasmid, LexA-P-dlg was co-transformed with a prey plasmid containing a human placenta cDNA library fused to the GAL4 activation domain in the pGAD GH vector (Clontech) by electroporation. The transformants (1×10^6) were screened with streaking on selection medium (SD-Trp, -Leu and -His) followed by β-galactosidase filter assay [24]. The pGAD GH plasmids containing the inserted cDNA were recovered from the positive clones and cotransformed with LexA-P-dlg or control baits to confirm their interaction. DNA sequencing of the cDNA inserted into the positive plasmid was performed by the dideoxynucleotide chain termination method.

3. Results

3.1. Identification of the human gene related to Drosophila dlg

To identify the human homolog of the *Drosophila dlg*, we performed sequence database searching using the Blast algorithm. A tblastn search of GenBank database using the dlg peptide sequences revealed that several EST clones have sequence similarity. Some of them had an identical sequence to the *PSD-95/SAP-90A* gene or the *p55* gene. The two potential overlapping ESTs, H29224 and H29225, were not found to be identical to any previously reported genes. Based on the sequences of these ESTs, we performed a two-step PCR to clone the full length cDNA. We identified a 2577 bp cDNA which contains an open reading frame encoding a polypeptide of 859 amino acids (Fig. 1).

A BLASTp search of the non-redundant protein database revealed that the predicted amino acid sequences of the cloned cDNA have a high similarity to a hdlg-1/SAP97 and *Drosophila* dlg with 45% and 40% identity, respectively. The P-dlg

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  1 MHASPPRKAR VRIASSYYPE GDGDSSHLPA KKSCDEDLTS
 41 QKVDELGQKR RRPKSAPSFR PKLAPVVIPA QFLEEQKCVP
 81 ASGELSPELQ EWAPYSPGHS SRHSNPPLYP SRPSVGTVPR
121 SLTPSTTVSS ILRNPIYTVR SHRVGPCSSP PAARDAGPQG
161 LHPSVQHQGR LSLDLSHRTC SDYSEMRATH GSNSLPSSAR
201 LGSSSNLQFK AERIKIPSTP RYPRSVVGSE RGSVSHSECS
241 TPPQSPLNID TLSSCSQSQT SASTLPRIAV NPASLGERRK
281 DRPYVEEPRH VKVQKGSEPL GISIVSGEKG GIYVSKVTVG
321 SIAHQAGLEY GDQLLEFNGI NLRSATEQQA RLIIGQQCDT
361 ITILAQYNPH VHQLSSHSRS SSHLDPAGTH STLQGSGTTT
401 PEHPSVIDPL MEQDEGPSTP PAKQSSSRIA GDANKKTLEP
441 RVVFIKKSQL ELGVHLCGGN LHGVFVAEVE DDSPAKGPDG
481 LVPGDLILEY GSLDVRNKTV EEVYVEMLKP RDGVRLKVQY
521 RPEEFTKAKG LPGDSFYIRA LYDRLADVEQ ELSFKKDDIL
561 YVDDTLPQGT FGSWMAWQLD ENAQKIQRGQ IPSKYVMDQE
601 FSRRLSMSEV KDDNSATKTL SAAARRSFFR RKHKHKRSGS
641 KDGKDLLALD AFSSDSIPLF EDSVSLAYQR VQKVDCTALR
681 PVLILGPLLD VVKEMLVNEA PGKFCRCPLE VMKASQQAIE
721 RGVKDCLFVD YKRRSGHFDV TTVASIKEIT EKNRHCLLDI
761 APHAIERLHH MHIYPIVIFI HYKSAKHIKE QRDPIYLRDK
801 VTQRHSKEQF EAAQKLEQEY SRYFTGVIQG GALSSICTQI
841 LAMVNQEQNK VLWIPACPL
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Fig. 1. Predicted amino acid sequence of the P-dlg (GenBank accession number U61843). On the basis of the deduced amino acid sequence, P-dlg contains 859 amino acids. Homologous domains and motifs are indicated as follows: the PDZ domains (box), the SH3 domain (solid underline) and the GUK domain (dashed underline).

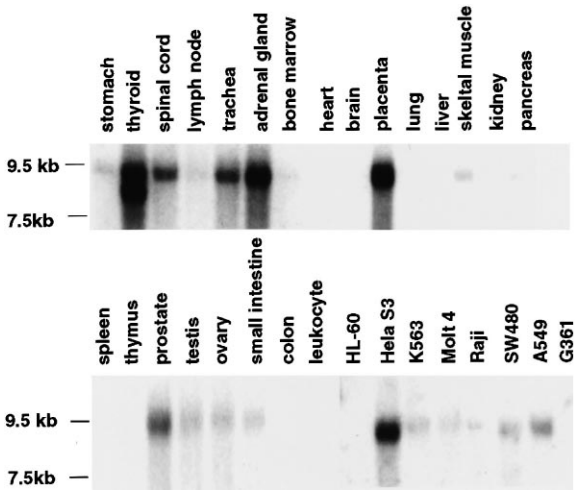

Fig. 2. Expression of *P-dlg* transcript in various human tissues and cancer cell lines. Each lane contains 2 μg of human poly(A)$^+$ RNA. Names of the tissues and cell lines are written at the top of the panel.

has three PDZ homology domains, the SH3 domain and the guanylate kinase homologous region (GUK). However, the first PDZ domain had weak homology compared to other PDZ domains. The GLGF motif conserved in most of mammalian dlg homologs was absent in all PDZ domains of P-dlg.

3.2. Expression of P-dlg mRNA in human tissues

To determine the expression pattern of *P-dlg* mRNA, we performed Northern blot analysis on various human tissues. *P-dlg* transcripts were detected in thyroid, spinal cord, trachea, adrenal gland, placenta and prostate. Since this gene was highly expressed in placenta and prostate, we designated it *P-dlg*. Thyroid was found to express two species (9.4 and 8.8 kbp) of *P-dlg* mRNA, and adrenal gland, trachea, spinal cord, placenta and prostate expressed only one of 9.4 kbp. Among the tumor cell lines examined, HeLa S3 cells highly expressed *P-dlg* transcript (Fig. 2).

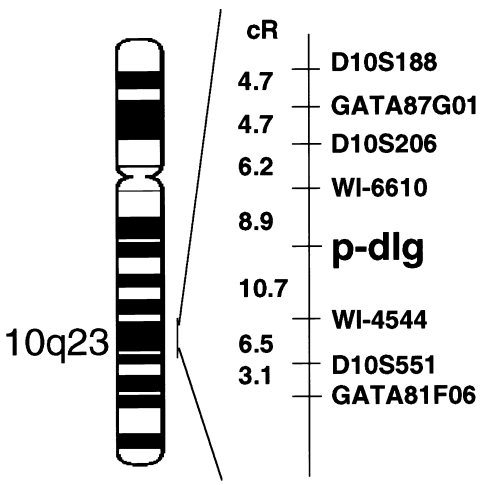

Fig. 3. Radiation hybrid mapping of the *P-dlg* gene. The results of PCR using the radiation hybrid panel were sent to Whitehead Institute/MIT Center for Genome Research via WWW and placed the P-dlg gene at 8.9 cR from WI-6610 on chromosome 10q23.

3.3. Chromosome localization of P-dlg gene

To identify the chromosome localization of the *P-dlg* gene, we performed PCR mapping using the Genebridge 4 radiation somatic cell hybrid panel. Comparison with the human chromosomal content of the hybrids, as determined by the manufacturer and Whitehead Institute/MIT Center for Genome Research, localized the *P-dlg* gene to chromosome 10q and placed it 8.88 cR from WI-7219 (Fig. 3).

3.4. Detection of the P-dlg protein by Western blot analysis

First, Western blot analysis of COS-7 cells, which were transiently transfected by the HA-tagged P-dlg expression plasmid (pCGN-*P-dlg*), was performed using anti-HA and anti-P-dlg antibodies. A band of about 105 kDa, which was very similar to the calculated molecular mass of the full length protein encoded by the P-dlg cDNA, was detected in lysates of pCGN-P-dlg transfected COS-7 cells with both anti-HA and anti-P-dlg antibodies (Fig. 4a). Using the P-dlg antibody, we performed Western blot analysis to detect the endogenous P-dlg protein in normal human prostate tissue and various human cancer cell lines (HeLa S3, PC-3 and Du-145). The 105-kDa protein was detected in lysates from human prostate tissues and HeLa S3 cells. However, the full length P-dlg protein was not detected in the prostate cancer cell lines PC-3 and Du-145 (Fig. 4b).

3.5. Immunodetection of P-dlg protein in prostate tissue and cultured cells

The *P-dlg* gene was found to be expressed in various endo-

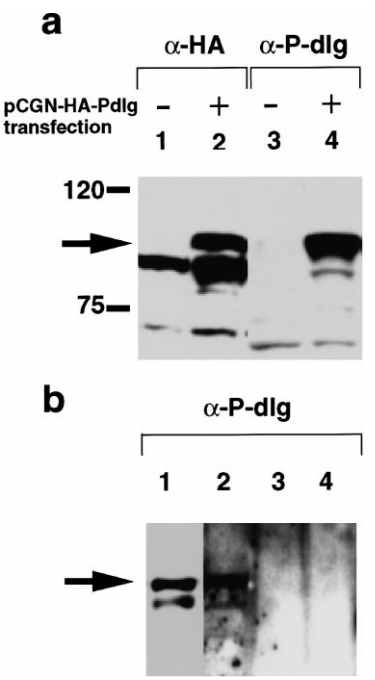

Fig. 4. Western blot analysis of P-dlg. a: Lysates prepared from COS-7 cells, which were transfected with pCGN-P-dlg (lanes 2 and 4) and with pCGN (lanes 1 and 3), were detected by anti-HA antibody (lanes 1 and 2) and by anti-P-dlg antibody (lanes 3 and 4). The arrow indicates the 105 kDa full length P-dlg protein. b: Lysates prepared from human prostate tissue (lane 1), HeLa cells (lane 2) and two human prostate cancer cell lines, PC-3 (lane 3) and Du-145 (lane 4) were detected by anti-P-dlg antibody. The arrow indicates the 105 kDa full length P-dlg protein.

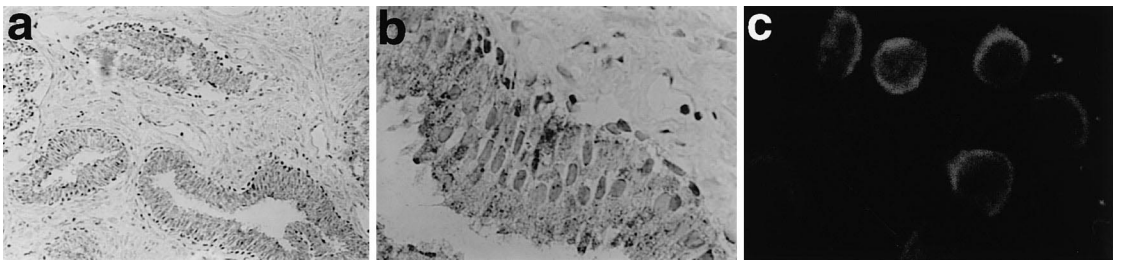

Fig. 5. Immunohistochemical analysis of P-dlg using anti-P-dlg polyclonal antibody. a, b: The gland epithelial cells of the normal human prostate tissue are specifically stained by anti-P-dlg antibody (frozen tissue section, (a) ×100, (b) ×400). c: Subcellular localization of the P-dlg protein in HeLa S3 cells. FITC positive staining is detected at the plasma membrane and the cytoplasm of HeLa S3 cells.

crine tissues including thyroid, adrenal and prostate. To determine the distribution of the P-dlg protein, immunohistochemical analysis using the anti-P-dlg antibody was performed in the frozen tissue sections of normal human prostate. P-dlg was found to be specifically expressed in the cytoplasm of the gland epithelial cells (Fig. 5a,b). We also examined localization of P-dlg protein in HeLa S3 cells by immunofluorescence staining followed by confocal microscopic analysis. FITC positive staining was detected along the plasma membrane and in cytoplasm of the cells (Fig. 5c).

3.6. Detection of an interaction between P-dlg and p55 proteins

To elucidate the function of the P-dlg protein, we performed yeast two-hybrid screening for identifying proteins that bind to the P-dlg protein. Five positive clones were obtained from 1×10^6 transformants screened. To confirm the specificity of the interactions, the plasmids expressing the activation domain fusion proteins were recovered from the positive clones and cotransformed with LexA-P-dlg and control baits. Three clones were positive in the secondary screening, and they contained a 1.2 kbp insert whose sequence was identical to a part of the p55 gene cDNA sequence. The insert encoded the carboxy-terminal 198 amino acids (269–466) of the p55 protein, which contains the GUK domain (Fig. 6).

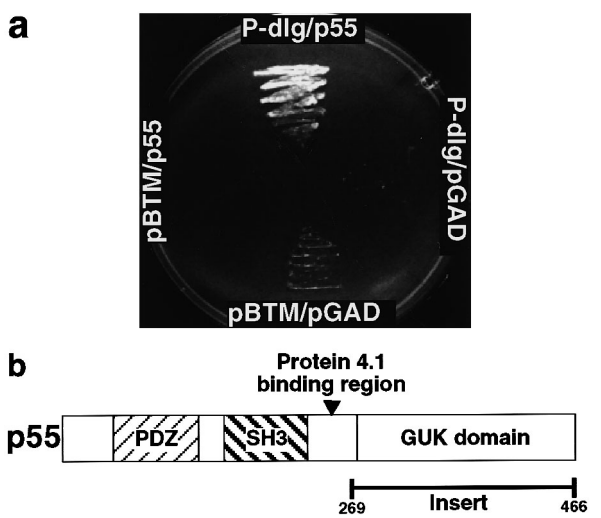

Fig. 6. Interaction of P-dlg with p55. a: Yeast two-hybrid assay of HIS3 reporter construct. The yeast transformants were streaked onto synthetic medium plates lacking tryptophan, leucine and histidine. Interaction between P-dlg and p55 resulted in activation of HIS3 reporter and enabled growth in the absence of histidine. b: Schematic representation of p55 gene product. The insert of the positive clone encodes the carboxy-terminal 198 amino acid fragment (269–466) of the p55 protein.

4. Discussion

We have identified and characterized the *P-dlg* gene, which is a novel human homolog of the *Drosophila dlg* tumor suppressor gene. The *P-dlg* transcript was highly expressed in placenta, prostate, thyroid, trachea, and adrenal gland, but not in brain. Additionally, immunohistochemical analysis of normal human prostate tissue revealed that the P-dlg protein is specifically expressed in the gland epithelial cells. This expression pattern of the *P-dlg* gene is different from other known human *dlg* homologs such as *h-dlg1/SAP97* and *NE-dlg/SAP102*; *h-dlg-1/SAP97* is ubiquitously expressed in most human normal tissues and *NE-dlg/SAP102* is expressed in brain and endocrine tissues [9].

The protein encoded by *P-dlg* gene has three PDZ domains, a SH3 domain and a GUK domain, which are conserved structures in some MAGUK family proteins. Among human MAGUK family proteins, p55, Dlg-2 and Dlg-3 have only one PDZ domain. In contrast, human SAP-90/PSD-95, hdlg-1/SAP-97, NE-dlg/SAP-102 and chapsyn 110 all have three PDZ domains and bind to the intracellular C-terminal tails of NMDA receptor subunits. The P-dlg protein has found to have three PDZ domains, however, the first (N-terminal) PDZ has weak homology compared to the other two PDZ domains. Furthermore, all three PDZ domains of the P-dlg do not possess the GLGF motif, which is conserved among most MAGUK family proteins and is necessary for the binding to the T/S-X-V motifs in the C-terminal peptides. InaD [25], which was identified as a molecule for clustering proteins at the photoreceptor cells, has the sequence Phe-Leu-Gly-Iso (FLGI) instead of the GLGF motif. Three cellular proteins which interact with PDZ domains of InaD protein were identified, but all these binding proteins lack the C-terminal T/S-X-V motif [26]. Therefore, P-dlg, similar to InaD, may not interact with the typical C-terminal T/S-X-V motifs. In fact, using the yeast two-hybrid system we confirmed that P-dlg does not bind to APC protein which has the C-terminal T/S-X-V motif (data not shown). Instead, we identified an association of P-dlg with the GUK domain of p55 by screening a prostate cDNA library employing the two-hybrid method. The C-terminal portion of p55 (289–466) has four internal T/S-X-V motifs which are possible binding sites for the PDZ domain of P-dlg.

p55 is a palmitoylated erythrocyte membrane protein, which is also one of the MAGUK family proteins. p55 has been previously shown to compose a ternary complex with the

band 4.1 protein and glycophorin C at the erythroid plasma membrane [20,21]. Genetic defects in either band 4.1 or glycophorin C result in the stoichiometric reduction of p55 protein and consequently induce aberrant morphology of erythrocytes and hemolysis. These lines of evidence indicate that this complex formation is involved in the cross-bridge system between the cytoskeleton and the plasma membrane in erythrocytes. Since isoforms of p55, band 4.1, and glycophorin C are present in many non-erythroid cells, these interactions are considered to modulate cytoskeletal-membrane linkage of broad cell types [19]. In fact, confocal microscopy findings in HeLa S3 cells have shown that the P-dlg protein is also localized at the plasma membrane and the cytoplasm. Taken together, our finding ssuggest that P-dlg and p55 form a heteromeric MAGUK complex at the plasma membrane and cluster various intracellular molecules to play roles in maintaining the structure of epithelial cells and transmitting extracellular signals to the membrane and cytoskeleton.

PCR mapping using the radiation somatic cell hybrid panel localized the *P-dlg* gene to chromosome 10q, 8.88 cR apart from WI-7219. The marker WI-7219 was mapped at chromosome 10q23, which was reported to be the frequent loss-of-heterozygosity region in prostate and endometrial tumors. Although P-dlg protein is abundantly expressed in normal prostate tissue, human prostate tumor cells (PC-3 and Du-145 cells) do not express P-dlg protein. These findings imply that the *P-dlg* gene is one of the candidate tumor suppressor genes in chromosome 10q23 and that a loss of function of the *P-dlg* gene may lead to the development of prostate tumor. Since *P-dlg* transcript was detected in the human prostate cancer cell lines examined (data not shown), detailed mutation analyses of the *P-dlg* gene will be required for an assessment of the involvement of *P-dlg* in the development of the tumors.

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